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Prediction of Antibiotic Influence by Presence of Some β-lactamase Gene in *Acinetobacter*Infected Patients Hospitalized in Tehran, Iran

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ABSTRACT

Acinetobacter baumannii which is gram-negative rod and infecting hospitalized patient often develops multiple drug resistance patterns and causes serious problem in treatment. The aim of this study is to determine the relationship between this serious problem in treatment and Presence of some β-lactamase genes in the *A. baumannii*. 100 isolates of Acinetobacter collected from hospitalized patients in Tehran. First their resistance to different antibiotics were determined using disk agar diffusion test then the MICs of all resistant isolates determined by Etest .In the second stage we screened all resistance isolates for β-lactamase gene existence by double-disk synergy test and finally prevalence of β-lactamase encoding genes such as IMP-1,SIM-1,OXA-23,OXA-24 and OXA-58 were determined by PCR. According those tests the most efficient treatment was achieved by Colistin, Gentamicin, and Imipenem. Disk synergy test with 0.1M EDTA determined 23 % of isolates as metalo β-lactamase producers. Prevalence of β-lactamase encoding genes was: 4%, 0%, 38%, 32%, and 1% respectively**.** According to this study efficient treatment will be influenced by OXA β-lactamase more than the other genes which have been studied. This is the first report of SIM type among MBL-producing *A. baumannii* in Iran.

KEY WORDS: Acinetobacter baumannii, β-lactamase, efficient treatment, PCR, Molecular typing.

INTRODUCTION

Acinetobacter baumannii is a gram-negative rod that causes an extensive range of serious infections such aspneumonia, sepsis and meningitis that frequently occurs in the strictly ill patients of ICU [1, 2] .There is a universal incessant rise in bacteremia incidence and mortality rate, mainly attributed to the increased usage of invasive devices [3].Risk factors are including antibiotic exposure, length of stay in ICU and basic disease. [2, 4].

Extensive use of empirical antibiotic chemotherapy has contributed to the appearance and increasing the number of *A. baumannii* strains resistant to a wide range of antibiotics, including broad-spectrum beta-lactams [5]. Recent reports also showed that the spread of the β-lactamase-mediated carbapenem resistance is the most common mechanism found in *A. baumannii* isolates carried out by the class B Metallo-β- lactamases (MBL) such as (IMP-1 and SIM-1 enzymes) or carbapenem-hydrolyzing class D β -lactamases (CHDLs) such as (OXA-23, 24, 51 and 58 -related families) [6, 7].MBLs and CHDLs have been identified worldwide from carbapenem-resistant *A. baumannii*strains.MBLs are powerful carbapenemases, and can hydrolyze a wide variety of β -lactams, including Penicillins, Cephalosporins,andCarbapenems [6, 7]. Infections are often difficult to treat since Carbapenems are now almost always the drug of choice for the treatment of *Acinetobacter* infections [8] and in some cases are associated with high morbidity and mortality rates [9].There are clear geographic differences in the prevalence of these mechanisms of β-lactamase resistance. [10, 11, 12].For this reason there have been extensive surveillance and research efforts worldwide focusing on the antibiotic susceptibilities [13, 14] and mechanisms of resistance [13, 15] of *A. baumannii* clinical isolates.

In Iran, our data on prevalence of β –lactamases gene and Correlation with antibiotic susceptibility pattern is very limited. In fact all the aim of this study is to acquire awareness about susceptibility patterns and Choice the best empirical therapy and more effective treatment in Tehran and probably other Persian Gulf countries's hospitalized patients.

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METHODS

Features of Study and Specimens. This is a cross-sectional study performed in 8 different hospitals from August 2009 to March 2010. The protocol of this study was accepted by the Technical Committee of the medical microbiology department of Shahed University. A total of 100 non-duplicate isolates of *A.baumannii* were collected from different clinical specimens of patients, who were hospitalized for ≥48 hours at different wards. Isolates were confirmed as *A. baumannii* by conventional biochemical testing and existence of gene encoding of OXA_{51}Amplicons. The isolated were then stored at –80°C in nutrient broth containing 50% glycerol v/v for further investigation.

Antimicrobial susceptibility test. Antimicrobial susceptibility was determined by disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) [16]. Minimum inhibitory concentrations (MICs) were determined by E-test (AB BIODISK, SOLNA, Sweden) according to the manufacturer's specifications. In brief for the E-test, 150-mm-diameter Muller-Hinton agar plates were inoculated with swabs saturated with suspensions of the isolates at 0.5 McFarland standards. The antimicrobial concentration ranges were: Piperacillin; 0.016- 256 µg/ml, Piperacillin/tazobactam; 0.016-256µg/ml, Ampicillin/sulbactam; 0.016- 256 µg/ml, Ciprofloxacin; 0.002 - 32 µg/ml, Ceftriaxone; 0.002-32 µg/ml, Cefotaxime 0.002-32 µg/ml, Ceftazidime; 0.016-256µg/ml,Cefepime; 0.016-256µg/ml, Colistin; 0.064-1024µg/ml, Tetracycline; 0.016-256 µg/ml, Imipenem; 0.016-256µg/ml, Amikacin; 0.016-256µg/ml and, Gentamicin; 0.016- 256 µg/ml. The results were read after 18 to 24 hours of incubation at 35°C. Quality control was performed using *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 strains.

Metalloβ -lactamase (MBL) producer screening. MBL-producing strains were screened with double-disk synergy tests (DDST) by placing 20 mm apart one Imipenem (10 µg) disk as substrate and another with 10µl 0.1 M EDTA (SIGMAChemical, ST. LOUIS, MO) as β-lactamase inhibitors. The zone around the Imipenem disk is extended on the side near the EDTA disk for a MBL producer [17].

DNA Extraction. DNA was extracted from the strains by boiling one to three colonies in 100 µl of sterile ultra-pure water for 10 min followed by centrifugation for 1 min at 14,000 rpm [18].

β-lactamase gene detectionby PCR. Primers used for amplification of blaOXA-51, blaOXA-23, blaOXA-24, blaOXA-58, IMP-1 and SIM-1 are given in Table 1**.**

Table 1. Oligonucleotides and other data used for detection of the β-lactamase genes

Target gene	Oligonucleotide	Sequence	Annealing Temperature (°C) &Time (Sec)	Size of Amplicons (bp)	Reference
blaOXA-51	OXA-51 F	5´-TAA TGC TTT GAT CGG CCT TG-3´	52,45	353	(18)
blaOXA-51	OXA-51 R	5´-TGG ATT GCA CTT CAT CTT GG-3'			
blaOXA-23	OXA-23 F	5´-GAT CGG ATT GGA GAA CCA GA-3'	52,45	501	(18)
blaOXA-23	OXA-23 R	5´-ATT TCT GAC CGC ATT TCC AT-3´			
blaOXA-24	OXA-24 F	5´-GGT TAG TTG GCC CCC TTA AA-3´	52,45	246	(18)
blaOXA-24	OXA-24 R	5´-AGT TGA GCG AAA AGG GGA TT-3´			
blaOXA-58	OXA-58 F	5´-AAG TAT TGG GGC TTG TGC TG-3´	52,45	599	(18)
blaOXA-58	OXA-58 R	5´-CCC CTC TGC GCT CTA CAT AC-3´			
blaSIM-1	SIM-1 F	5´-TAC AAG GGA TTC GGC ATC G-3´	43,30	570	(19)
blaSIM-1	SIM-1R	5´-TAA TGG CCT GTT CCC ATG TG-3´			
blaIMP-1	IMP-1F	5´-AAC CAG TTT TGC CTT ACC AT-3´	43,30	188	(19)
blaIMP-1	IMP-1R	5´-CTA CCG CAG CAG AGT CTT TG-3´			

PCR was performed with 5µL of heat-extracted DNA template, 1 µl of each primer, 3 µldNTP, 1.25µL $Mgcl_2$ 25mM and 3µL of*Taq* DNA polymerase in a total volume of 30 µl. A Master cycler instrument (TECHNETouch gene Gradient Thermal Cyclers - Krackeler Scientific Inc.UK.) was used with the following reaction conditions: 94°C for 2min 35 cycles , 94°C for 1 min, 43-52°C for 30-45 Sec, and 72°C for 90 sec, and finally, 72°C for 7 min**.** PCR products were separated by electrophoresis on a 1% agarose gel and were detected by ethidium bromide staining and comparison against a 100 bp DNA ladder as a size marker under the visualization of UV light.

Statistical analysis. Collected data analyzed in SPSS V 16 software using descriptive statistical methods such as table, graph and mean+/- SD. Universal analysis was conducted using student's t test, ANOVAs for quantitative and chi-square for categorical variables.

RESULTS

A total of 100 samples were obtained. Patients' medical records were referred to extract clinical information such as age (n=2;0-20 years, n=38;21-40 years, n=33;41-60 years,n=24;61-80 years,n= 3;81-100 years) , sex (men; n=50,women;n=50) and hospital location. The organisms were isolated from blood (n=8), urine (n=7), cerebral spinal fluid (CSF) (n=3), Sputum (n=8), pleura fluid (n=10), Wound (n=34), Catheter (n=7)Broncho alveolar lavage (BAL) (n=22), Synovial fluid (n=1). The majority of isolates were obtained from patients in intensive care units (n=54), Burn (n=18), Urology (n=4), Orthopedic (n=2) and Internal medicine (n=22) wards. The most important site for obtained of *A. baumannii*was Wound and the most frequent ward of hospital was intensive care unit(54%) followed by Internal (22%), Burn (18%), Urologic (4%), Orthopedic Wards. (2%).Distribution of antimicrobial resistance among *A. baumannii* isolates at 8 different hospitals of Tehran is shown in Table 2.

Table 2. Distribution of antimicrobial resistance among *A.baumannii* isolates at 8 differenthospitals of Tehran

Hospital / Antibiotic	H1 n=26	H2 n=17	H3 n=14	H4 n=10	H5 n=9	H6 n=9	H7 n=8	H8 n=7	p-value
Piperacillin	26(100%)	17(100%)	14(100%)	10(100%)	9(100%)	9(100%)	8(100%)	7(100%)	0.0018
Piperacillin/ Tazobactam	24(92.3%)	15(88.2%)	12(85.7%)	7(70%)	9(100%)	9(100%)	6(75%)	7(100%)	0.002
Ampicillin-sulbactam	15(57.6%)	14(82.3%)	7(50%)	8(80%)	5(55.5%)	3(33.3%)	5(62.5%)	5(71.4%)	0.013
Ciprofloxacin	26(100%)	17(100%)	13(92.8%)	9(90%)	7(77.7%)	8(88.8%)	6(75%)	7(100%)	0.0002
Ceftriaxone	26(100%)	17(100%)	14(100%)	10(100%)	7(77.7%)	8(88.8%)	7(87.5%)	7(100%)	0.0004
Cefotaxime	26(100%)	17(100%)	14(100%)	10(100%)	8(88.8%)	8(88.8%)	8(100%)	7(100%)	0.0009
Ceftazidime	26(100%)	16(94.1%)	13(92.8%)	9(90%)	8(88.8%)	8(88.8%)	6(75%)	7(100%)	0.0003
Cefepime	25(96.1%)	17(100%)	14(100%)	10(100%)	9(100%)	9(100%)	8(100%)	6(85.7%)	0.0024
Colistin	5(19.2%)	0(0%)	0(0%)	1(10%)	1(11.1%)	0(0%)	2(25%)	0(0%)	0.18
Tetracycline	20(76.9%)	12(70.5%)	7(50%)	7(70%)	5(55.5%)	4(44.4%)	4(57.1%)	7(100%)	0.0009
Imipenem	11(42.3%)	9(52.9%)	10(71.4%)	6(60%)	3(33.3%)	3(33.3%)	5(62.5%)	6(85.7%)	0.19
Amikacin	13 (50%)	13(76.4%)	8(57.14%)	7(70%)	4(44.4%)	5(55.5%)	7(87.5%)	5(71.4%)	0.13
Gentamicin	8(30.7%)	10(58.8%)	6(42.8%)	5(50%)	3(33.3%)	3(33.3%)	5(62.5%)	5(71.4%)	0.42

H1;Motahhari,H2;Imam Hossain, H3;Millad,H4;Mustafa,H5;Imam Khomeini,H6;Loghman,H7;Labbafinejad,H8;Khatamanbia.

Distribution of antimicrobial resistance among *A. baumannii* isolates according to different sample origins is shown in Table 3.

Table 3. Distribution of antimicrobial resistance among *A. baumannii* isolates according todifferent sample origins

Origin / Antibiotic	Wound n=34	Synovialfluid n=1	Sputum n=8	Blood n=8	BAL n=22	Pleura fluid n=10	Catheter n=7	Urine n=7	CSF n=3	p-value
Piperacillin	34(100%)	1(100%)	8(100%)	8(100%)	22(100%)	10(100%)	7(100%)	7(100%)	3(100%)	0.001
Piperacillin/ Tazobactam	30(88.2%)	0(0%)	8(100%)	5(62.5%)	22(100%)	10(100%)	7(100%)	5(71.4%)	2(66.6%)	0.002
Ampicillin-sulbactam	20(58.8%)	1(100%)	6(75%)	5(62.5%)	17(77.2%)	6(60%)	1(14.2%)	5(71.4%)	1(33.3%)	0.003
Ciprofloxacin	34(100%)	1(100%)	7(87.5%)	7(87.5%)	21(95.4%)	9(90%)	6(85.7%)	5(71.4%)	3(100%)	0.001
Ceftriaxone	33(97.0)	1(100%)	8(100%)	8(100%)	22(100%)	9(90%)	7(100%)	5(71.4%)	3(100%)	0.001
Cefotaxime	34(100%)	1(100%)	8(100%)	8(100%)	22(100%)	10(100%)	7(100%)	5(71.4%)	3(100%)	0.001
Ceftazidime	31(91.1%)	1(100%)	8(100%)	6(75%)	22(100%)	10(100%)	6(85.7%)	6(85.7%)	3(100%)	0.004
Cefepime	33(97%)	1(100%)	8(100%)	8(100%)	22(100%)	10(100%)	7(100%)	6(85.7%)	3(100%)	0.001
Colistin	3(8.8%)	0(0%)	3(37.5%)	0(0%)	2(9%)	0(0%)	0(0%)	1(14.2%)	0(0%)	0.74
Tetracycline	28(82.3%)	0(0%)	6(75%)	6(75%)	14(63.6%)	4(40%)	4(57.1%)	2(28.5%)	2(66.6%)	0.001
Imipenem	15(44.1%)	1(100%)	4(50%)	5(62.5%)	15(68.1%)	5(50%)	3(42.8%)	4(57.1%)	1(33.3%)	0.0024

From among all antibiotics Colistin, Imipenem, Amikacin and Gentamicin have not statistically significant difference in various hospitals but other antibiotics have significant difference (Table 2).From among all antibiotics Colistin has not statistically significant difference in Various sample origins but other antibiotics have significant difference (Table 3).

The most effective antimicrobial agents against *A. baumannii* isolates among the antimicrobials tested were Colistin, Gentamicin and then Imipenem. This study indicated high level resistance in *Acinetobacter baumannii* strains isolate (Figures1-2).

Of 100 isolates of *A. baumannii*, 23% were found to be producing MBL by DDST.*β-lactamase* gene of isolates gave PCR products of various sizes ranging from 188 bp to 599 bp (Table 1) and(Figures3-4).Based on this evaluation, PCR detecting *β-lactamase* gene showed as follow: OXA-51 n=100(100%), OXA-58 n=1(1%), OXA-23 n=38(38%), OXA-24 n=32 (32%), IMP-1 n=4(4%) and finally SIM-1 n=0(0%). All isolates harbored OXA-51,Whereasthere was not any Ampicillin-sulbactam resistant isolates harboring OXA-58, However, Ceftriaxone and Cefotaxime resistant isolates were the most common OXA-23 harboring isolates (30.2%,29.5%) respectively. Cefotaxime and Ceftazidime resistant isolates were the most common OXA-24 harboring isolates (32.6%, 31.1%) respectively. Imipenem resistant isolates were the most common IMP-1 harboring isolates (7.5%). The relationship between antibiotic resistance and the existence of different *β-lactamase* gene is shown in Table 4. The association between drug resistance to all antibiotics and the presence of β-lactamase gene had statistically significant difference (Table 4).

Table 4. The relationship between antibiotic resistance and the existence of different *β-lactamase* gene in isolates of *A. baumannii*

Antibiotic	Number of Resistant isolate	Gene presence						p-value
		OXA-51 n (%)	OXA-58 n (%)	OXA-23 n (%)	OXA-24 n (%)	IMP-1 n (%)	SIM-1 n (%)	
Piperacillin	100	100(100)	1(1)	25(25)	29(29)	4(4)	0 (0)	0.001
Piperacillin/ Tazobactam	89	89(100)	1(1.1)	16(17.9)	23(25.8)	2(2.2)	0 (0)	0.001
Ampicillin/ Sulbactam	62	62(100)	0(0)	16(25.8)	14(22.5)	2(3.2)	0 (0)	0.002
Ceftriaxone	96	96(100)	1(1.0)	29(30.2)	25(26)	3(3.1)	0 (0)	0.0012
Cefotaxime	98	98(100)	1(1.0)	29(29.5)	32(32.6)	3(3.0)	0 (0)	0.001
Ceftazidime	93	93(100)	1(1.0)	25(26.8)	29(31.1)	3(3.2)	0 (0)	0.003
Cefepime	98	98(100)	1(1.0)	19(19.3)	19(19.3)	4(4.0)	0 (0)	0.001
Imipenem	53	53(100)	1(1.88)	11(20.7)	10(18.8)	4(7.5)	0 (0)	0.005

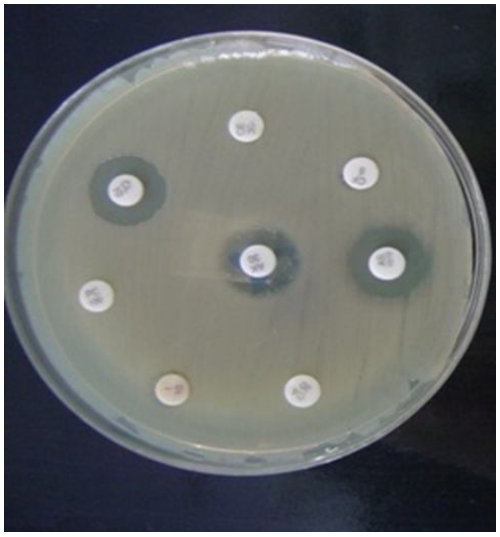

Fig. 1.*A. baumannii*, clinical isolates Antibiogram; disk diffusion method.

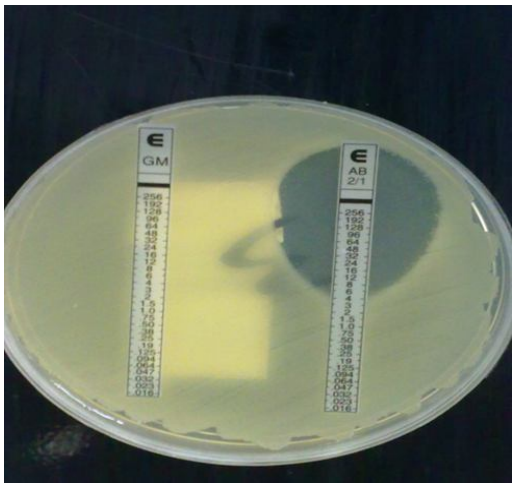

Fig. 2. Minimum inhibitory concentrations by E-test
(AB BIODISK, SOLNA, Sweden).

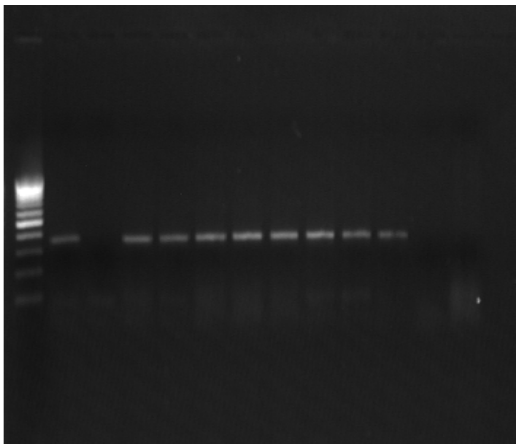

Fig. 3. PCR of OXA-51 among clinical
A. baumannii isolates Lane 1 (DNA Ladder 100bp). Lane 2(Positive control) Lane 3(Negative control)
Lanes 4-11 (clinical positive isolates).

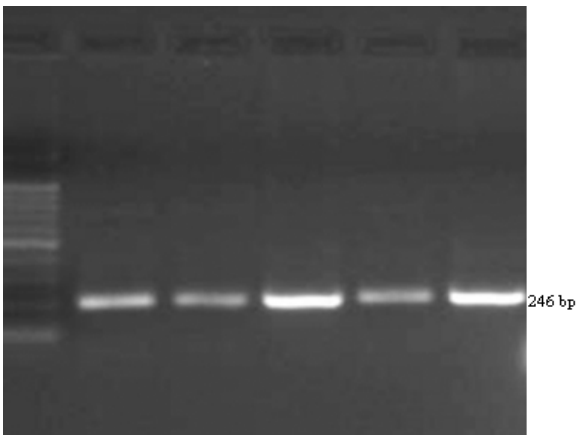

Fig. 4.PCR of OXA-24among clinical *A. baumannii*isolates.Lane 1 (DNALadder 100 bp). Lane 2 (Positive control),Lanes 3-6
(clinical positive isolates).

DISCUSSION

Unfortunately due the lack of updated and efficient information about antibiotic susceptibility pattern and high Capacity of the *A.baumannii*, this Opportunistic organisms gain the powerful resistance to the most of drugs Treatment. In this study we attempt to gain effective and sufficient information about antibiotic resistance pattern and presence of some β-lactamase genes. In fact the aim of this study is to increase the augury power of failing or successes of treatment.

In the current study, most samples were isolated from the wound (34%) and the other important source was bronco alveolar lavage (BAL=22%).In the studies which have been reported from Iran and other countries the Most of samples were isolated from the Wounds [18, 20, 21], tracheal tube aspiration [22]. and respiratory system [17]. This Similarity between the origins of samples, Is due to the fact that the injured tissue because of burning or using invasive device such as ventilator is appropriate location for growing microbe.

Our study results are in agreement with reports from the other study which showed high prevalence rate of *A.baumannii* isolation in ICU [20, 2]. This high incidence may be due to the use of aggressive therapeutic agent in these wards.

Now the three most effective antibiotics against *Acinetobacter* were found to be Colistin, Gentamicin and Imipenem. Our study results are in agreement with the other report from Tehran that has shown low resistance to the Colistin and Imipenem.[23]. In past years low resistance rates of *A. baumannii* to Imipenem (about 3%) were reported from Saudi Arabia and Japan ,4.5% from Tehran Hospitals and 9.6% from Turkey [22] but now this Resistance rate is 53% in this study, 43.7% in Turkey and 43% in Spain [22].On the other hand our study results are in agreement with the reports from the other countries that have shown high resistance to the most of the prescribed antibiotics such as CTX, CAZ, CIP, SXT (90–100%) in Germany [24] and Cephalosporins in Europe (15% to 97%) [25].Regional variation in resistance of *A. baumannii* to Imipenem and other antibiotics is related to the previous pattern of antimicrobial use and risk factors such as duration of hospital and ICU stay, usage of the endotracheal tube, venous catheter and urinary catheter.

In this study, the class D β -lactamases were detected more frequently than MBLs thus *bla*OXA-51 harbored in all Isolates and *bla*OXA-58, *bla*OXA-23 and *bla*OXA-24 genes were detected in1, 38 and 32 isolates respectively, Whereas *bla*IMP-1 and *bla*SIM-1 were detected only in 4 and 0 isolates respectively. Our results regarding blaOXA-51 were in agreement with other studies [26],.In one study in Colombia Sixty five of the 66 isolates were positive for *bla*OXA-23 genes and all 66 were positive for *bla*OXA-51 genes, Whereas all of them were negative for *bla*OXA-58 and blaOXA-24 genes [27].In another published study all of 1018 isolates were positive by PCR for *bla* OXA-23- and blaOXA-51 genes in China Whereas no IMP, SIM-1, OXA-58 or OXA-24 genes were detected. [28]. In one study in Korea only 7 strains from 31 isolate harbored *bla*OXA-23, 15 isolates had *bla*IMP whereas there was not any OXA type β -lactamase gene, including *bla*OXA-24 , and *bla*OXA-58 [6].In the recent study in Iran 19 isolates from 100 isolates carried the *bla*IMP gene [16]. These various results may suggest that very mechanisms or genes have contributed to the Carbapenem resistance in these isolates (e.g., reduced affinity of penicillin-binding proteins and decreased permeability of the outer membrane).

Our results also indicate that there is a significant statistical difference between resistance to some antibiotics, hospitals, different wards of hospitals and beta lactamase genes. At the present time, the most effective drug for treatment of patients is usage of Colistin, Gentamicin and Imipenem.

Considering the results of this research, it seems that we can predict the drug resistance or lack of it, but it is necessary to gain updated and complete information regarding genes and resistance mechanisms with continual molecular monitoring of resistance pattern which finding of this research, are a small effort to achieve the above mentioned goal.

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